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Title: **The optimal oral biopsy site for diagnosis of mucous membrane pemphigoid and pemphigus vulgaris**

Running title: The optimal oral biopsy site for diagnosis of MMP and PV

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What's already known about this topic?

- The variation in sensitivity of oral biopsy sites for direct immunofluorescence (DIF) in the diagnosis of oral MMP and PV has not been studied in detail in large series of patients.
- Biopsy can be challenging due to difficult access and fragility of the oral mucosa. The diagnostic biopsy technique is therefore critical.

What does this study add?

- We have shown that a normal buccal punch biopsy (NBPB) from uninvolved oral mucosa is as sensitive as a perilesional biopsy (PLB) for diagnosis of oral PV, and superior to serology and histology.
- For multisite MMP, while the PLB has the highest sensitivity, the NBPB is almost equivalent for diagnosis and is more sensitive than serology and histology.

What are the clinical implications of this work?

- Oral punch biopsy technique on uninvolved buccal mucosa tissue is a simple and safe practical method for diagnosing oral PV and MMP.

Abstract

Introduction: Accepted 'standard practice' for the diagnosis of immunobullous disease (IBD) is a perilesional sample for direct immunofluorescence (DIF). Our aim was to compare diagnostic outcomes of a normal buccal punch biopsy (NBPB) with a perilesional biopsy (PLB) for mucous membrane pemphigoid (MMP) and pemphigus vulgaris (PV).

Method: A retrospective analysis of 251 DIF positive MMP and 77 DIF positive PV patients was undertaken. Parameters analysed included the intraoral sites of involvement, histopathological, DIF and indirect immunofluorescence (IIF) findings.

Results: For MMP, PLB was positive in 134/143 (93.7%) samples compared with 129/144 (89.6%) NBPB. The diagnostic sensitivity for PLB and NBPB (81.3% (39/48) and 77.1% (37/48), respectively, among 48 patients who underwent both techniques and was not statistically significant (p=0.62). In gingival-only MMP, PLB was positive in 63/69 (91.3%) and NBPB was positive in 63/75 (84.0%). Among multisite MMP, PLB was positive in 71/74 (95.9%) and NBPB was positive in 66/69 (95.7%). In gingival-only MMP, biopsies from reflected alveolar mucosa in 17 consecutive patients were positive in 17/17 cases (100%). For PV, PLB was positive in 42/43 (97.7%) compared with 42/42 (100%) NBPB. Histopathology

was diagnostic in 93/134 (69.4%) MMP and 38/41 (92.7%) PV. IIF was positive in 126/197 (63.9%) MMP and 68/74 (91.9%) PV patient sera.

Conclusion: In the largest series of combined oral DIF results in MMP and PV, we have shown that a NBPB is almost equivalent as a PLB for the diagnosis of PV and multisite MMP and is more sensitive than both histology and IIF.

Introduction

Autoimmune bullous diseases (AIBD) represent a heterogeneous group, characterised by immunoreactivity to structural proteins that maintain cell-cell and cell-matrix adhesion in the skin and mucous membranes.[1] Desmoglein 1 and 3 are considered the main target antigens in pemphigus vulgaris (PV). In mucous membrane pemphigoid (MMP), the main target antigens proposed include bullous pemphigoid antigen 2 (BPA2/ BP180), bullous pemphigoid antigen 1 (BPAg1/ BP230), laminin 332, alpha6 beta4 (α6β4) integrin and less commonly, collagen VII.[2,3,4] The oral cavity is affected in 84–96% of mucous membrane pemphigoid cases.[2,5,6] The gingiva is the most commonly affected site (80%)[7], followed by the buccal mucosa and palate, and less commonly, the tongue and lips. A subgroup of patients present with desquamative gingivitis (DG) as the sole manifestation (64% of cases),[8] whilst other patients present with involvement of parts of the oral mucosa without DG, and in a third group all of the oral mucosa may be affected.[9] In MMP, blisters may be present on the gingiva or palate.

In PV, the oral mucosa is the first site of involvement in the majority of cases and the disease may remain confined to the mucosal surfaces or extend to involve the skin.[10,11,12] Any site within the oral mucosa may be involved, however, the buccal mucosa, labial mucosa, ventrolateral tongue, and soft palate, all non-keratinised and readily traumatised, are especially vulnerable. The typical findings in both disorders are ulceration or erosions that may be covered by a yellow-white fibrinous slough. Gingival lesions usually include severe desquamative gingivitis, in which bullae have ruptured, to leave peeling tissue with red erosions or deep ulcers on the attached gingiva[13]. DG is the presenting feature in 21% of PV cases.[8] Blisters are much less commonly seen; however small haemorrhagic blisters are sometimes seen on the buccal mucosa. As the mouth is very trauma prone, more usually, erythema or ulcers are present. Imminent blistering or conversely mucosal healing are both associated with a white appearance of the tissue and this still reflects disease activity.

The diagnosis of MMP and PV involving the oral cavity is based upon a combination of clinical, histopathological analysis and immunopathologic findings.[11,14] Early diagnosis and treatment are essential to reduce complications. Direct immunofluorescence (DIF) is particularly helpful

in distinguishing PV from the subepidermal blistering diseases, though circulating autoantibodies detected by indirect immunofluorescence (IIF) or enzyme-linked immunosorbent assay (ELISA) may act as a surrogate where a biopsy is unavailable.[7,15,16,17,18] The accepted 'standard practice' however for diagnosis of MMP and PV, is laboratory evidence of an antibody-mediated disease at the epithelial basement membrane zone for MMP and the surface of epidermal keratinocytes for PV. This is accomplished by either a perilesional punch biopsy or incisional biopsy for DIF.[14,19,20,21,22,23] For lesions in the mouth, an oral punch biopsy offers a convenient and practical method of confirming oral diagnosis. A recent study concluded punch biopsy was more sensitive than scalpel biopsy for suspected autoimmune bullous disease.[24] The buccal mucosa can be exposed by everting the cheek, placing the thumb at the commissure and reflecting the corner of the mouth, applying external pressure on the cheek with the index finger to present the buccal mucosa.[25]

Previous studies have reported sensitivity rates of 70%-80% using DIF for MMP[7,22,23,26] and 90-100% for PV[17,19,26,27,28,29] using either perilesional or a buccal punch biopsy technique (Table 1). Selection of the optimal site for biopsy is important as the diagnostic ability of DIF depends on the presence of intact epithelium along all or most of the specimen. This can only reliably be achieved by sampling normal-appearing mucosa, combined with careful surgical techniques and minimal handling of the tissue.[29] Obtaining an intact gingival biopsy in cases presenting with desquamative gingivitis alone is often difficult because the mucosa is thin, often friable and difficult to process. Hence inadequate surgical technique, sub-optimal surgical site selection, or improper tissue handling may lead to the loss of the gingival epithelium.

Indirect immunofluorescence (IIF) is helpful in confirming a suspected diagnosis as well as differentiating closely related subepidermal bullous diseases by identifying their binding pattern. Assay of serum antibody titres by IIF may also help guide prognostication and therapy. In MMP, IIF studies for IgG and /or IgA are positive in approximately 50-84% of cases.[7,30,31,32] Patients with high IgG titres are more likely to need systemic management, and virtually all those with both circulating IgG and IgA require systemic immunosuppressive therapy.[11] In PV, almost all patients with active disease have detectable IgG autoantibodies and titres tend to correlate with disease activity.[33,34] Because the detection of circulating autoantibody by IIF is inconsistent overall, DIF remains the preferred method for diagnosis.[35,36]

The variation in sensitivity of oral biopsy sites for DIF in the diagnosis of oral MMP and PV has not been studied in detail in large series of patients. The aim of the study was to compare the diagnostic sensitivity of an uninvolved 4mm NBPB with a PLB for MMP and PV and to ultimately make recommendations for the optimal sites to sample.

Methods

All patients had been referred to either the Oral Medicine department or St John's Institute of Dermatology, Guy's and St Thomas' Hospital, London. A retrospective analysis of 251 DIF positive MMP and 77 DIF positive PV patients was performed. All patients were positive on at least one biopsy sample. Patients were biopsied routinely for diagnostic purposes using either a perilesional site for DIF, an unaffected site for DIF or both and histopathology. The potential value of an unaffected site had been previously shown in a small study in our department (REC 95/304) and based upon these results we had incorporated this additional biopsy site into our routine clinical practice.

In this analysis and service review, we collated the data on both intraoral and extraoral sites of involvement, histopathological findings, DIF results from PLB and/or NBPB and IIF. Biopsy specimens for DIF microscopy were placed in Michel's transport medium and referred to the laboratory. Michel's transport medium (pH 7.0–7.2), was used to ensure that any specimens not reaching the lab for up to 28 days could be safely stored. It is associated with remarkable preservation of all components of the basement membrane zone.[37] Normal saline may be used as an alternative if the biopsy sample can be shipped to the laboratory within 24 hours.[38] PLBs were defined as those taken within a radius of 1 cm of a clinical lesion, and NBPB as clinically normal uninvolved buccal mucosa (Figure 1). Individual sections were incubated with fluorescein isothiocyanate-labelled antibodies against immunoglobulin (Ig)G, IgA, IgM, complement 3, and fibrinogen, and examined using a fluorescence microscope as previously described[39,40]. Indirect IF was undertaken as previously described and used monkey oesophagus and human skin for detecting pemphigus autoantibodies.[39] EDTA-split skin was used to distinguish between different subepidermal bullous diseases.[39,41] Commercial enzyme-linked immunosorbent assays (ELISAs) for the detection of antibodies to desmoglein (Dsg) 1 (Dsg antibody values >30U/ml = positive), Dsg3 (antibody values >30U/ml = positive), BP180 NC16a (antibody values >20U/ml = positive) and BP230 (antibody values >10U/ml = positive) were utilised. No other domains of BP180 have been tested nor has reactivity with laminin 332 as these are not yet commercially available. All ELISA kits used were manufactured by Medical and Biological Laboratories (MBL) Co. Ltd, Nagoya, Japan. The threshold values used for positivity

were 30 U/ml (DSG1 and 3), 20 U/ml (BP180) and 10 U/ml (BP230), derived from analysis of sera from a UK blood donor population. The BP180 ELISA kits used detect antibodies binding to a recombinant, purified full-length NC16A domain of BP180 only. Although the soluble ectodomain of BP180 contains a significant portion of the NC16A domain, the additional pathogenic domains of BP180 in this fragment are not assessed by the MBL ELISA. The values for sensitivity were calculated with the standard formula: sensitivity = true-positives/(true-positives + false-negatives). The diagnostic sensitivity and DIF positivity between PLB and NBPB were tested using proportions test.

Results

Mucous Membrane Pemphigoid

A total of 251 DIF positive patients were identified among whom a total of 304 biopsy samples for immunofluorescence were analysed (Figure 2). One hundred and forty-three underwent PLB, 144 underwent NBPB, 48 underwent both PLB and NBPB and 17 underwent reflected alveolar mucosa biopsies. DIF was positive in 134/143 (93.7%) PLB samples and 129/144 (89.6%) NBPB samples. Forty-eight patients had DIF performed on both PLB and NBPB. Sensitivity was similar in these samples (p=0.62); 81.3% (39/48) for PLB versus 77.1% (37/48) for NBPB.

One hundred and thirty-three (53.0%) patients had gingival-only (desquamative gingivitis only) oral disease. Of these cases, PLB was positive in 63/69 (91.3%) and negative in 6 samples. NBPB was positive in 63/75 (84.0%) of cases and negative in 12. One hundred and eighteen (47.0%) patients had multisite oral disease (any site of the oral mucosa may be affected, with or without gingival involvement). Of these, PLB was positive in 71/74 (95.9%) and NBPB was positive in 66/69 (95.7%). In contrast to those with gingival-only disease where 6/69 (8.7%) PLB and 12/75 (16%) NBPB biopsies were negative, in multi-oral site MMP only 3/74 (4.1%) PLB samples and 3/69 (4.3%) NBPB of multisite oral disease cases were negative. On review of the 3 cases with multisite disease and DIF negative PLB, the tissue was either heavily inflamed (1 sample) or there was loss of epithelium (2 samples). In the patients with DIF negative NBPB and multisite oral disease, all three patients had predominantly gingival disease with palatal involvement, with no buccal mucosa affected. Latterly, we have undertaken perilesional reflected alveolar mucosa in consecutive gingival-only MMP and demonstrated positive DIF in all 17 cases (100%). In those who underwent NBPB in addition to perilesional adjacent reflected alveolar mucosa, DIF was negative in 4/5 (80%) of NBPB samples.

Histopathology was available for 134 patients and was diagnostic in 93/134 (69.4%). Indirect IF was positive in 126/197 (63.9%) of cases. A dual circulating anti-basement membrane zone (anti-BMZ) antibody response with IgG and IgA was detected in 22.9% (28/122 cases). Antibodies to IgG were detected in 72/122 (59.0%) and to IgA in 18.0% (22/122). Anti-basement membrane zone antibodies were detected to the following titres: 64.2% (77/122) had a titre of 1/10, 20.5% (25/122) a titre of 1/100, 11.5% (14/122) a titre of 1/200, 0.8% (1/122) a titre of 1;400, 2.5% (3/122) a titre of 1/800 and 1.64% (2/122) a titre of 1/1600. Circulating autoantibodies against BP180 alone using standard commercial ELISA kits (antibody values >20U/ml = positive) were detected in 12/58 patients (20.6%), circulating autoantibodies against BP230 (antibody values >10U/ml = positive) in 6/58 (10.3%) patients and circulating antibodies to both in 9/58 (15.5%). In those who underwent PLB, autoantibodies to BP180 alone were detected in 0/16 (0%), to BP230 alone in 1/16 (6.2%) and to both in 3/16 (18.6%) Overall 25% patients had detectable circulating BP autoantibodies. In NBPB cases, autoantibodies against BP180 alone were detected in 8/27 (29.6%), against BP230 alone in 3/27 (11.1%) and to both in 4/27 (14.8%). In this subgroup 15/27 (55.6%) patients had detectable circulating BP autoantibodies. Of the 48 patients undergoing both biopsy techniques, autoantibodies against BP180 were detected in 4/15 (26.7%), against BP230 in 2/15 (13.3%) and against both BP180 and 230 in 2/15 (13.3%). Here 8/15 (53.3%) patients had detectable BP autoantibodies. Our patients have not been tested so far for anti-laminin 332 autoantibodies.

Pemphigus Vulgaris

A total of 77 DIF positive PV patients were identified and a total of 85 samples were analysed. (Figure 3). DIF was positive in 42/43 (97.7%) PLB samples and in 42/42 (100%) NBPB samples and the difference was not statistically significant (p=0.32). Eight cases had DIF performed on both PLB and NBPB. PLB was positive in seven and negative in one. NBPB was positive in all cases. Histopathology was diagnostic in 38/41 (92.7%) patients. IIF was positive in 68/74 (91.9%) of cases. Circulating autoantibodies (antibody values >30U/ml = positive) against desmoglein 3 alone were detected in 35/60 (58.3%), against desmoglein 1 alone in 1/60 (1.7%), and circulating autoantibodies against both desmoglein 1 and 3 were detected in 19/60 (31.7%) of cases.

Discussion

An accurate diagnosis of PV and MMP is necessary in order to plan appropriate treatment protocols and define patient prognosis. The gold standard for the diagnosis is DIF[14,19-23], however controversy exists regarding the optimal oral biopsy site for DIF studies.

In the largest reported series to date of combined oral DIF results in MMP and PV, we have shown that a NBPB from uninvolved oral mucosa is as sensitive as a PLB for the diagnosis of PV and multisite MMP. Among 251 MMP patients, DIF was positive in 133/141 (94.2%) PLB samples and 129/144 (89.6%) NBPB samples and the difference was not statistically significant (p=0.14). The sensitivity was similar in the 48 patients who had DIF samples from both sites (p=0.62); 81.3% (39/48) for PLB versus 77.1% (37/48)) for NBPB though marginally more sensitive in PLB. Among 118 patients with multisite oral MMP, there was no difference in sensitivity between PLB positive in 71/74 (95.9%) and NBPB positive in 66/69 (95.7%). Among 77 PV patients, there was minimal difference between PLB 42/43 (97.7%) vs NBPB 42/42 (100%) patients. Thus for PV, either site was highly reliable. Therefore, in patients with PV or multisite MMP, these data suggest that autoantibodies bind in-vivo throughout much of the oral mucosa.

The same may not be true for pure gingival MMP. There are technical issues to consider when the inflammation is localised to the gingiva. MMP may present with desquamative gingivitis where the gingivae are often friable, fiery red, painful, and eroded, seen primarily on the labial or buccal aspect.[8,42,43,44] The attached gingiva is a band of keratinized mucosa that stretches from the neck of the tooth to the reflected alveolar mucosa and is tethered to the underlying periosteum and bone. Establishing a positive diagnosis from a gingival sample can be technically difficult and importantly, from a long term oral health perspective, will result in a periodontal defect leading to an area subsequently prone to plaque accumulation and periodontal disease.[30] The gingival tissue in active disease is also fragile and thin and easily detached from the underlying connective tissue, which leads to loss of epithelium and potentially false negative results.[45] A number of studies in addition to our own, have demonstrated gingival biopsies to be an inferior biopsy site that is also prone to non-specific inflammatory changes in that area.[21,26,29] Our data have shown that gingival-only MMP accounted for the negative results in 6/9 (66.7%) PLB and 12/15 (80%) NBPB. Thus, DIF sensitivity was lower in both PLB (91.3% vs 95.9%) and NBPB (84.0% vs 95.7%) for gingival vs multisite patients, respectively. If lesions are present at several oral mucosal sites including the gingiva, it is therefore preferable to avoid the gingiva. Based upon these data and the finding that a PLB from 17 consecutive patients of reflected

alveolar mucosa was positive on DIF in gingival-only patients, we advocate a biopsy from this site, being perilesional but avoids loss of the papilla and loss of epithelium (Figure 4).

When DIF is unavailable, assay of serum antibody titres by IIF may be helpful for diagnosis, acting as a surrogate in some patients, it may also be helpful as a prognostic indicator. In this study, IIF was positive in 91.9% of PV cases and 63.9% of MMP cases. The detection of circulating autoantibodies by IIF was inconsistent and titres were variable. We have shown that a NBPB was superior to IIF for both PV and MMP. In MMP, circulating autoantibodies against BP180 alone were detected in 12/58 patients (20.6%), against BP230 in 6/58 (10.3%) patients and circulating antibodies to both in 9/58 (15.5%). A dual circulating anti-basement membrane zone (anti-BMZ) antibody response with IgG and IgA was detected in 28/122 (22.9%), antibodies to IgG in 72/122 (59.0%) and to IgA in 22/122 (18.0%). Circulating autoantibodies against desmoglein 3 alone were detected in 35/60 (58.3%) PV patients and circulating autoantibodies against both desmoglein 1 and 3 were detected in 19/60 (31.7%) i.e. overall Dsg3 antibodies were detected in 90% patients. These findings are consistent with data published elsewhere.[46,47,48] Once commercially available ELISAs are available for the C terminal domain of BP180 and laminin 332, it is possible that diagnostic sensitivity will be significantly enhanced for MMP.

Light microscopic (histopathological) studies are not considered an absolute criterion for the diagnosis of MMP[14] or PV[18]. In this study, oral histology was diagnostic in 92.7% of PV cases and 69.4% of MMP cases where this investigation was undertaken. For MMP, histology of lesional tissue typically demonstrates subepithelial blisters with or without leukocyte infiltration. The characteristic findings in PV include intraepithelial cleavage with acantholysis primarily localised to the suprabasal region with retention of basal keratinocytes along the basement membrane zone, resulting in an appearance that resembles a "row of tombstones". Combining the data from this study, NBPB was superior to histopathological examination for both PV and MMP. Although histological analysis generally should be performed, especially where the diagnosis includes other pathology such as lichen planus, it is expected that DIF will remain a gold standard in diagnosing autoimmune bullous disease.

A number of studies have investigated the sensitivity of DIF in oral PV and MMP biopsies (table 1). Jordan et al[49] proposed that all patients with active oral PV should have a positive DIF for IgG on the cell surface of epithelial cells. Several studies have reported the sensitivity of DIF for oral MMP to be less sensitive.[21-23] DIF sensitivity was 92% in oral PV and 78% in oral pemphigoid in

a study by Helander and Rogers[21] and 89% in oral PV and 68% in oral pemphigoid in a study by Rogers and van Hale.[50] Some authors[27] have proposed that when investigating skin autoimmune diseases, that biopsies from normal-appearing tissue 5-10mm away from clinically apparent skin lesions might yield the best DIF test results, avoiding loss of immunoreactants by the inflammatory process in lesional samples. Many studies have described immunoreactants potentially present in the entire affected oral mucosal surface[26] and in the unaffected oral mucosa.[2] Sano et al reported no significant difference in the DIF detection rate between oral biopsies taken perilesionally (66.1%) or distant from the clinical lesion (64.7%).[26] However their sample size for immunobullous cases was relatively small (n=125). The reasons for the variability in DIF sensitivity rates are suggested to be poor biopsy site selection (a site too close to or too distant from lesions), technical difficulties in sampling, diagnostic laboratory technical and reporting skills poorly accessible mucosal sites or uneven distribution of immunoreactants in the mucosa.[51] In the oral cavity, chronic inflammation is particularly common around the gingivae which may partially account for the lower sensitivity of biopsies from this site.

Diagnostic delay is very common when PV or MMP are confined to the oral mucosa.[52,53] It is important to emphasize the significance of obtaining a good biopsy specimen, as the diagnostic ability of DIF depends on the presence of attached epithelium along all or most of the specimen. A specimen in which the epithelium has completely separated from the connective tissue is non-diagnostic for subepidermal IB diseases and is extremely unreliable for PV as well. This separation can usually be avoided by sampling normal-appearing or erythematous perilesional mucosa, with a gentle surgical technique and minimal handling of the tissue. This study provides evidence that the oral punch biopsy technique on uninvolved buccal mucosa tissue is a simple and safe practical method for diagnosing oral PV and MMP. The technique requires no additional instruments or special skills, is technically easy and has high diagnostic value. In addition, the NBPB technique is less traumatic for the patient and heals without scarring.

Conclusion

In the largest reported series of combined oral DIF results in MMP and PV, we have shown that a NBPB from uninvolved oral mucosa is as sensitive as a PLB for diagnosis of oral PV, superior to serology and histopathology though both also have high sensitivities. For multisite MMP, while the PLB has the highest sensitivity, the NBPB is almost equivalent for diagnosis and is more sensitive than histology and IIF. For pure gingival MMP, neither a PLB from the attached gingiva nor NBPB are sensitive enough to be individually reliable. Combining the two increases

sensitivity however, two biopsies for DIF increases both morbidity and cost. Our data has shown that a biopsy from reflected alveolar mucosa adjacent to an area of active full thickness gingival inflammation provides 100% sensitivity. For specialists not fully trained in complex oral biopsy techniques, a NBPB appears to be a good alternative technique to a PLB for all but pure gingival IB disease. It is a simple, reliable, sensitive technique and can be used by a variety of clinicians including dermatologists and ophthalmologists.

Figure legends:

Figure 1: Perilesional (black circle) and normal buccal punch (yellow circle) biopsy sites

Figure 2: DIF results in MMP. DIF was positive in 134/143 (94%) PLB samples and 129/144 (90%) NBPB samples. DIF was positive in 17 (100%) of perilesional reflected alveolar mucosa biopsies in gingival-only MMP.

Figure 3: DIF results in PV. DIF was positive in 42/43 (97.7%) PLB samples and in 42/42 (100%) NBPB samples and the difference was not statistically significant (p=0.32).

Figure 4: In patients with lesions confined to the gingiva, a biopsy should be taken from adjacent normal reflected alveolar mucosa.

Table 1: Data from previous studies: diagnosis, site of active disease, biopsy site and result. MMP= mucous membrane pemphigoid, PV= pemphigus vulgaris, OLP= oral lichen planus, LAD= linear IgA disease, PV= pemphigus vulgaris, EM= erythema multiforme.

Study	Number of cases	Number of biopsies undertaken	Diagnosis	Site of active disease	Biopsy site (Skin/oral cavity)	Results
Gilvetti et al, 2018	66	125	OLP (24) MMP (20) PV (4) Bullous pemphigoid (1)	Oral: 66 Skin: 23 Ocular: 8 Pharyngeal: 7 Genital: 7	Oral: Perilesional	Biopsies for DIF taken in 45/66. Diagnosis AIBD (histo and DIF) in 49/66 (74%).
Kamaguchi et al, 2018	7	7	MMP	Oral: 6 gingival only, 1 multi-site	Oral: Non-lesional buccal mucosa	DIF +ve 100% (7/7) IIF -ve 100% (7/7) Histology +ve 14% (1/7)
Shimanovich et al, 2017	78	138	MMP	Oral:68 Skin:20 Genital:6 Ocular: 29 Nasal: 18 Pharynx: 2 Larynx: 1 Oesophagus: 1	Oral:94 Skin:28 Genital:2 Ocular: 14	DIF +ve 95% (74/78)
Endo et al, 2014	27	52	MMP (13) PV (8) OLP (6)	Oral: gingival	Oral: Perilesional	MMP: DIF: +ve (16/24) Histo: +ve (10 /26) PV: DIF: +ve (8/24) Histo: +ve (8 /26) OLP: DIF: +ve (0/24) Histo: +ve (3 /26) Non-specific: Histo: 5/26

Suresh et al, 2012	239	396	OLP (106) MMP (60) PV (5) LAD (2)	Oral: Gingival	Oral: Gingival	DIF +ve: OLP 44.3% (106/239) MMP 25.1% (60/239) Pemphigus 2.1% (5/239) LAD 0.8% (2/239) Other 5.4% (13/239) Non-diagnostic/ negative: 22% (53/239)
Sano et al, 2008	125	129	OLP (52) Cicatricial pemphigoid (14) Bullous pemphigoid (5) PV (9)	Oral mucosa: Not defined	Oral: Perilesional (<1cm from lesion) Distant (>1cm from lesion)	DIF Distant +ve 64.7% DIF PLB +ve 66.1% DIF +ve: OLP 65.8% (52/79) Cicatricial pemphigoid 66.7% (14/21) Bullous pemphigoid 55.6% (5/9) PV 100% (9/9)
Setterfield et al, 1998	67	126	MMP	Oral 94% (63/67) Ocular 93% Skin 36% Nasopharynx 34% Genitalia 28%	Oral: 59 perilesional Skin: 24 perilesional Conjunctival: 43 perilesional or non-lesional	DIF +ve overall 95.5% (64/67) IIF +ve 83.5% (56/67)
Helander et al, 1994	500	500	Cicatricial pemphigoid (69) Bullous pemphigoid (2) MMP (10) PV (21)	Oral	Oral: Perilesional	PV: DIF +ve 89% (18/21) Pemphigoid: DIF +ve: 68% (55/81) OLP: DIF +ve 61% (108/178)

			OLP (178)			
Siegel at al, 1993	5	5	Cicatricial pemphigoid	Oral (5) Ocular (1) Nasal (1)	All non-lesional Oral: 4/5 Ocular: 1/5	Cicatricial pemphigoid: DIF +ve 80% (4/5) LAD: DIF +ve (20% (1/5)
Daniels et al, 1981	130	130	MMP (33) PV (10) OLP (59) EM (22) Lupus erythematous (6)	MMP: Oral 32/33 Oral and skin 1/33 PV: Oral 2/10 Oral and cutaneous 8/10	Oral: Perilesional	MMP DIF +ve 97% (32/33) Histology 76% (25/33) PV DIF +ve 100% (10/10) Histology +ve 100% (10/10)
Laskaris, 1981	58	58	PV (58)	PV: Oral only	Oral: Perilesional	DIF +ve 98.3% (57/58) Histo: +ve 93.1% (54/58)
Laskaris et al, 1981	33	66	Cicatricial pemphigoid (33)	Oral 32 Skin 1 Genitals: 1 Nasal: 2 Larynx: 7 Ocular:10	Oral: Perilesional (33) Skin (33): Non-lesional	Histo: Oral: 75.7% (25/33) DIF: +ve Oral: 96.9% (32/33) Skin: 3% (1/33)
Siegel at al, 1993	5	5	Cicatricial pemphigoid	Oral (5) Ocular (1) Nasal (1)	All non-lesional Oral: 4/5 Ocular: 1/5	DIF +ve 80% (4/5) LAD: DIF +ve (20% (1/5)

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Figure 1:

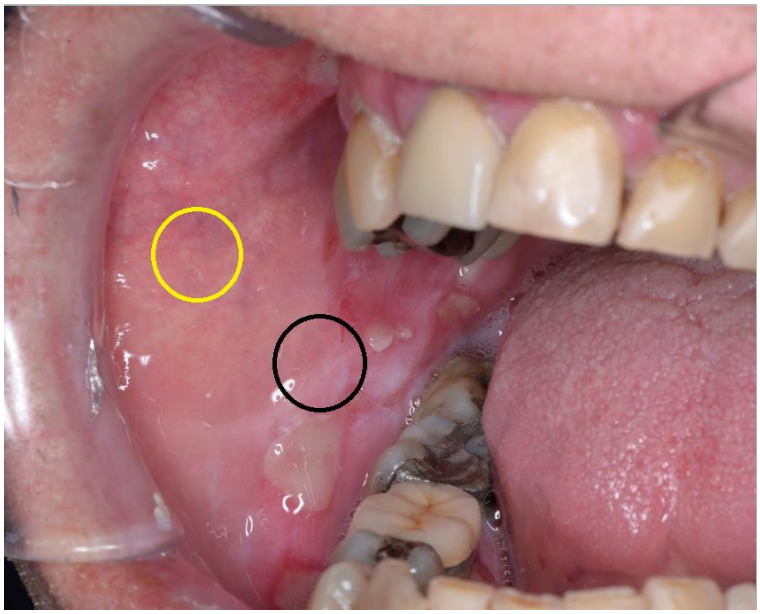

Figure 2:

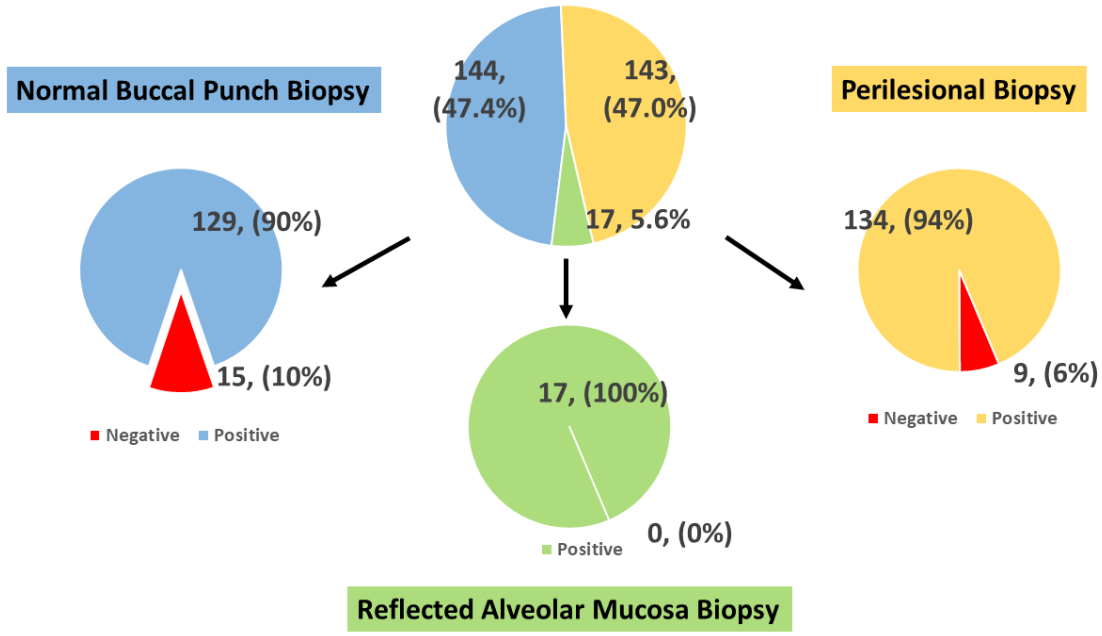
Normal Buccal Punch Biopsy
144, (47.4%)
143, (47.0%)
Perilesional Biopsy
129, (90%)
17, 5.6%
134, (94%)
15, (10%)
9, (6%)
Negative
Positive
Negative
Positive
17, (100%)
0, (0%)
Positive
Reflected Alveolar Mucosa Biopsy

Figure 3:

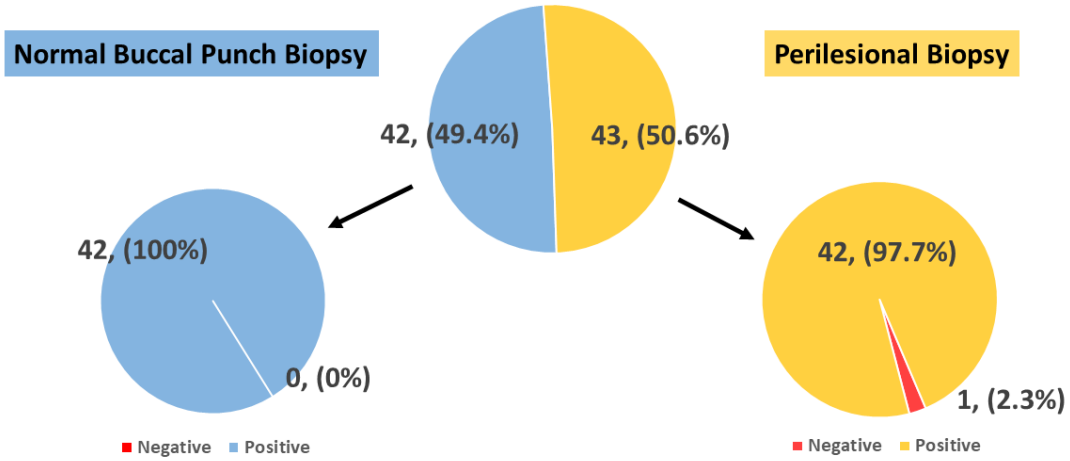
Normal Buccal Punch Biopsy
42, (49.4%)
43, (50.6%)
Perilesional Biopsy
42, (100%)
42, (97.7%)
0, (0%)
1, (2.3%)
Negative
Positive
Negative
Positive

Figure 4: